(51) International Patent Classification 5 :	(11) International Publication Number: **WO 92/19⁹**
G01N 21/76 **A2**	(43) International Publication Date: 12 November 1992 (12.11

(54) Title: METHOD FOR OPTICALLY MEASURING CHEMICAL ANALYTES

(57) Abstract

A system and method in which a photoluminescent ligand is added to a sample to be analyzed in the form of a photolᵤminescent probe having intrinsic analyte-induced lifetime changes. The method preferably employs phase-modulation fluoromₑtry to measure the lifetime changes. Specific probes are disclosed for measuring various analytes, particularly ionic solutes, inⱼcluding H^+, Ca^{2+} and K^+.

METHOD FOR OPTICALLY MEASURING CHEMICAL ANALYTES

FIELD OF THE INVENTION

The present invention is directed to a method for optically measuring chemical analytes and, more particularly, to a method in which a luminescent ligand is added to the sample to be analyzed in
5 the form of a photoluminescent probe having intrinsic analyte-induced lifetime changes.

BACKGROUND OF THE INVENTION

Measurement of certain analytes in blood, such as pH and carbon dioxide, is an important aspect of the clinical care of
10 patients. Previously, such measurements have been made using gas chromatography and other chemical methods. These methods are disadvantageous in that it is necessary to ship the blood sample to a clinical laboratory for analysis, which often results in a delay of an hour or more. Moreover, since the blood gases change
15 rapidly, the shipping time may cause the results to be invalid. Furthermore, these known methods cannot be used for continuous in-vivo monitoring of blood.

It is known to optically measure certain analytes by using fluorescence intensity measurements. Although fluorescence
20 intensity measurements are desirable in their simplicity, such measurements suffer from source fluctuations due to noise, drift and the like, and are subject to fluorophore bleaching, probe wash-out and background fluorescence. Further, if the media is turbid or colored, the intensity measurements will be greatly affected.
25 Moreover, since intensity is a linear product of numerous factors, such as the amount of fluorophore in each state, the excitation intensity, the excitation and emission bandpass, the wavelength sensitivity of the detector, and the like, a complex set of calibration curves must be used to accommodate these factors.

SUMMARY OF THE INVENTION
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The present invention overcomes the above difficulties by providing a method in which a luminescent ligand is added to the sample to be analyzed in the form of a photoluminescent probe having intrinsic analyte-induced lifetime changes. The lifetime

measurements are advantageous over intensity measurements because they can be performed in optically dense samples or turbid media and are independent of and/or insensitive to photobleaching, probe wash-out or optical loss. The lifetime changes are measured using known time-resolved or phase-modulation fluorometry methods.

In accordance with the method of the invention, the probe can be either fluorescent, wherein the emission is from an excited singlet state to a ground state and the rate of return to the ground state occurs rapidly (on the order of 10 ns), or phosphorescent, wherein the emission is from an excited triplet state to a singlet state and the rate of return to the ground state is relatively slow (on the order of msec to sec).

The step of adding a luminescent ligand (i.e., probe) to the sample to be analyzed requires matching a particular probe to a particular analyte, so that at least a portion of the sample will be non-covalently bound to the probe so that both bound and unbound species of the probe will exist. Thus, the invention differs from prior lifetime measurement methods which rely on a collisional quenching mechanism for measuring analytes. See, for example, U.S. Patent No. 4,810,655 to Khalil et al.; and Great Britain Patent No. 2,132,348 to Demas et al.

By definition, in collisional quenching, the probe does not bind to the analyte as required by the present invention. Instead, collisional quenching requires collisional contact between the fluorophore (probe) and the quencher (analyte). For collisional quenching to occur, the quencher must diffuse to interact with the fluorophore while the latter is in the excited state. Thus, the excited fluorophore returns to the ground state without emission of a photon.

In contrast, the present invention may have an "enhancement" of the luminescence. When the fluorescent ligand binds to the analyte, there may be an increase or decrease in intensity. It is also to be emphasized that the method of the present invention is not a Forster energy transfer mechanism, and thus is different from the method disclosed in European Patent Application 397,641 to Wolfbeis.

The method is useful for sensing the pH and carbon dioxide concentration in blood and other bodily fluids. To this end,

particular probes have proven to be particularly suitable includin
seminaphthofluoresceins, seminaphthorhodafluors and resorufins
including those sold by MOLECULAR PROBES of Eugene, Oregon, unde
the tradenames SNAFL-1, Carboxy SNAFL-1, Carboxy SNAFL-2, BCEC:
5   Acid, Carboxy SNARF-1, Carboxy SNARF-2, Carboxy SNARF-6 and Carboxy
SNARF-X, and resorufin derivatives such as the sodium salt and the
acetate.

The method of the invention is useful in either in vitro or
in vivo applications, including, for example, blood gas catheters,
10 including optical fibers, and other bedside monitors, and non-
invasive blood gas measurements. Also, the invention may be used
for pH and carbon dioxide sensors for fermentors and incubators.

The method of the invention is not limited to the sensing of
pH, but may also be used to sense a wide variety of other ionic
15 solutes. For example, the calcium ion concentration of a sample
may be determined by the method of the present invention. Sensing
the calcium ion concentration of a sample is particularly useful
for flow cytometry and fluorescent lifetime imaging applications.
Such applications are generally disclosed in U. S. Application
20 Serial No. 07/595,343, entitled Method and Apparatus for Performing
Phase Fluorescence Lifetime Measurements in Flow Cytometry, filed
October 10, 1990, and U. S. Application Serial No. 07/645,525,
entitled Method and Apparatus for Multi-Dimensional Phase
Fluorescence Lifetime Imaging, filed January 24, 1991, the contents
25 of which are incorporated herein by reference. Of course, the
method of the invention is generally applicable to flow cytometry
and fluorescent lifetime imaging, and such applications are in no
way limited to just calcium ion sensing.

Certain tetraacetic acid derivatives such as that sold by
30 Molecular Probes under the tradename Quin-2 are particularly useful
calcium ion probes, although it is contemplated that other probes,
such as those sold under the tradenames Fura-2, Indo-1, Rhod-2 and
Fluo-3 may also prove suitable. Also, the calcium ion indicators
sold by Molecular Probes under the trade-names Calcium Green,
35 Calcium Orange and Calcium Crimson may be used.

Further, the method of the invention may be used to measure
potassium ion concentration. Certain potassium ion indicators
including that sold by Molecular Probes under the name PBFI may be

useful as potassium ion probes in the method of the invention.

It is to be noted that, although these various pH, calcium ion and potassium ion indicators are known commercially, these indicators generally heretofore have not been used for quantifying
5   their corresponding analytes by photoluminescence lifetime measurements. Most recently, the probes Fura-2 and Quin-2 have been used to make fluorescent lifetime measurements in the presence and absence of calcium ions. See, respectively, S. M. Keating, "Nanosecond Fluorescence Microscopy of Single Cells", report of
10 proceedings mailed May 3, 1990; and Miyoshi et al, "A New Method of Determining Intracellular Free Ca^{2+} Concentration Using Quin2-Fluorescence", Photochemistry and Photobiology, Vol. 53, No 3, pp. 415-418 (1991).

BRIEF DESCRIPTION OF THE DRAWINGS
15   Figure 1 is a phase/modulation versus pH graph for one of the probes, snafl-1, used in a preferred embodiment of the invention;

        Figure 2 is a phase/modulation versus pH graph for another of the probes, carboxy snafl-1, used in another preferred embodiment of the invention;
20   Figure 3 is a phase/modulation versus pH graph for another of the probes, carboxy snafl-2, used in another preferred embodiment of the invention;

        Figure 4 is a phase/modulation versus pH graph for another of the probes, BCECF acid, used in another preferred embodiment of
25 the invention;

        Figure 5 is a phase/modulation versus pH graph for another of the probes, carboxy snarf-1, used in another preferred embodiment of the invention;

        Figure 6 is a phase/modulation versus pH graph for another
30 of the probes, carboxy snarf-2, used in another preferred embodiment of the invention;

        Figure 7 is a phase/modulation versus pH graph for another of the probes, carboxy snarf-6, used in another preferred embodiment of the invention;
35   Figure 8 is a phase/modulation versus pH graph for another of the probes, carboxy snarf-X, used in another preferred embodiment of the invention;

Figure 9A is a phase versus pH graph for another of the probes, resorufin sodium salt, used in another preferred embodiment of the invention;

Figure 9B is a modulation versus pH graph for the probe shown 5 in Figure 9A;

Figure 10 is a phase/modulation versus pH graph for another of the probes, resorufin acetate, used in another preferred embodiment of the invention;

Figure 11 is a phase/modulation versus calcium ion 10 concentration graph for another of the probes, Quin-2, used in another preferred embodiment of the invention;

Figure 12 is a phase/modulation versus frequency graph for the probe in Figure 5;

Figure 13A is a phase versus concentration graph for carbon 15 dioxide for the probe, carboxy SNARF-6, used in another preferred embodiment of the invention;

Figure 13B is a modulation versus concentration graph corresponding to the graph shown in Figure 13A;

Figure 14 is a phase versus concentration graph for potassium 20 ion concentrations for another of the probes, PBFI, used in another preferred embodiment;

Figure 15 is a schematic diagram showing an embodiment of the preferred instrumentation for use in the present invention;

Figure 16 is another phase/modulation versus pH graph for the 25 probe shown in Figure 3; and

Figure 17 is another modulation versus pH graph for the probe shown in Figure 3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The method in accordance with a preferred embodiment of the 30 invention determines and quantifies chemical analytes by changes in photoluminescence lifetimes. The method of the invention includes adding a luminescent ligand to the sample containing the analyte to be analyzed in the form of a photoluminescent probe. The probe can be either fluorescent or phosphorescent.

35   It is to be understood that this step requires matching a particular probe to a particular analyte, so that at least a portion of the analyte will become non-covalently bound to the

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probe so that both bound and unbound (i.e., free) species of the probe will then exist within the sample. The probe is chosen to have intrinsic analyte-induced lifetime changes, i.e., when the probe is bound to an analyte, the naturally occurring fluorescent

5 or phosphorescent.lifetime changes. It is to be understood that throughout this application the term "lifetime" refers to the photoluminescent lifetime defined as the inverse of the decay rate of the probe. These changes in lifetime can be measured to determine the concentration of the analyte, as will become more

10 apparent from the discussion below.

In the context of the present invention, the term "sample" refers to compounds, surfaces, solutions, emulsions, suspensions, mixtures, cell cultures, fermentation cultures, cells, tissues, secretions and/or derivatives or extracts thereof, as well as

15 supercritical fluids. Samples, as defined above, which can be used in the method of the present invention for sensing analytes based on fluorescence lifetimes also include samples that can be clear or turbid. Such samples to be measured according to the present invention require only that the fluorophore used be contacted with

20 the sample such that the analyte to be sensed influences the lifetime of the fluorophore such that the lifetime varies with the presence or amount of the analyte.

Such samples can also include, e.g., animal tissues, such as blood, lymph, cerebrospinal fluid, bone marrow, gastrointestinal

25 contents, and portions, cells or internal and external secretions of skin, heart, lung and respiratory system, liver, spleen, kidney, pancreas, gall bladder, gastrointestinal tract, smooth, skeletal or cardiac-muscle, circulatory system, reproductive organs, auditory system, the autonomic and central nervous system, and extracts or

30 cell cultures thereof. Such samples can be measured using methods of the present invention _in vitro_, _in vivo_ and _in situ_.

Such samples can also include environmental samples such as earth, air or water samples, as well as industrial or commercial samples as compounds, surfaces, aqueous chemical solutions,

35 emulsions, suspensions or mixtures.

Additionally, samples that can be used in the method of the present invention include cell culture and fermentation media used for growth of prokaryotic or eukaryotic cells and/or tissues, such

as bacteria, yeast, mammalian cells, plant cells and insect cells

The term "analyte" in the context of the present inventio refers to elements, ions, compounds, or salts, dissociatio products, polymers, aggregates or derivatives thereof. Example

5 of analytes that can be measured in the method of the presen invention include, e.g., H^+, Ca^{2+}, K^+ or other compounds containing these ionic solutes, including salts, derivatives, polymers, dissociation products, or aggregates thereof.

The method of the invention further includes exciting the
10 tagged sample with radiation from any suitable radiation source, such as a laser, an light emitting diode or the like. Light sources suitable for use in the methods of the present invention, also include noble gas light sources such as helium, neon, argon, krypton, xenon, and radon, and combinations, thereof. Light
15 sources can include gas lamps or lasers which provide uniform light that has been filtered, polarized, or provided as a laser source, such as a coherent wave (CW) laser or a pulsed laser. Specified impurities can be added to the above described noble gas light sources to provide suitable light sources for use in the present
20 invention with varying wavelengths such as emission and excitation wavelengths. Such impurities include Group II metals, such as zinc, cadmium, mercury, strontium, selenium and ruthenium. A green helium-neon laser is particularly preferred in the present invention because it is inexpensive and reliable.

25   In a preferred embodiment, the intensity of the excitation radiation is modulated at a particular modulation frequency and the lifetime determined using known phase-modulation, i.e., frequency-domain, techniques. Alternatively, a pulsed radiation source may be used and the lifetime of the sample determined using
30 known time-resolved methods. Both phase-modulation and time-resolved fluorometry methods are well known in the prior art, see Lakowicz, _Principles of Fluorescence Spectroscopy_, Plenum Press, 1983, Chapter 3. However, current instrumentation renders the phase-modulation method more expedient. For the sake of
35 conciseness, only the phase-modulation method will be discussed further herein, but it is understood that these same principles generally apply to time-resolved measurements.

When the sample is excited with radiation whose intensity is

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modulated, for example, in a sinusoidal manner, the time lag between absorption and emission causes the emission to be delayed in phase and demodulated relative to the excitation radiation. As discussed above, when a luminescent ligand is added to the sample, at least a portion of the analyte will bind with the ligand, i.e., probe, so that both bound and unbound species of the probe will now exist within the sample. The probe is chosen so that there will be a significant difference in the luminescent lifetime between the bound and unbound species. The phase shift ϕ and the corresponding demodulation factor m can be measured and used to calculate the photoluminescent lifetime based on well known formulae. See, Lakowicz, supra. It is desirable to select the modulation frequency in a range that coincides with the frequency at which the differences between the measured phase angles and the demodulations of the bound and unbound ligand are maximal.

Thus, according to the method of the invention, the emission radiation is detected, the phase shift ϕ (in degrees) and the demodulation factor m (as a percentage change) are measured, and the apparent photoluminescent lifetime may be calculated therefrom. An absolute value of difference in phase angle between the bound and free forms of the ligand of the order of 30-60 degrees at some preselected frequency, and a difference in modulation factor on the order of 30-87%, are preferred, which is shown in the examples below. These ranges of phase angles and modulation factors are preferred because they offer the greatest precision and dynamic range.

The absolute values of the frequency-dependent phase differences and demodulations are determined by the photoluminescent lifetimes of the free and bound ligand. In addition, if the excitation and emission spectra are not congruent, the effects depicted in Figure 16 can occur, whereby at particular wavelengths of excitation or emission one form or the other of the probe is preferentially excited or its emission preferentially observed. In such cases, the apparent analyte concentration (for pH, the apparent pKa) varies with excitation or emission wavelength. This is advantageous, in that the method of the present invention allows the range of concentrations that can be accurately measured with a single probe to be easily varied by

selection of the appropriate excitation and/or emission wavelengths.

One preferred embodiment of the instrumentation for use with the method of the invention is schematically shown in Figure 15.
5 It is to be understood, however, that any suitable instrumentation can be used, including, for example, those disclosed in U. S. Patent No. 4,937,457 to Mitchell, and those disclosed in Lakowicz, "A Review of Photon-Counting and Phase-Modulation Measurements of Fluorescence Decay Kinetics", Applications of Fluorescence in the
10 Biomedical Sciences, pp. 29-67 (1986), the contents of which are incorporated herein by reference.

As shown in Figure 15, radiation source 10, in this case a helium-neon laser having an emission of 543 nm, emits excitation beam 12 which is modulated by acoustooptic modulator 14 at a
15 frequency f1 to create sinusoidally-modulated excitation beam 16. It is to be understood that modulator 14 need not be an acoustooptic modulator, but that any suitable modulator may be used, such as an electrooptic modulator. Moreover, the modulation need not be sinusoidal, but of any desired shape. Also, the
20 modulator need not be external, but instead the light source may be intrinsically modulated.

Sinusoidally-modulated excitation beam 16 irradiates sample S, which contains the analyte to be measured and the appropriate probe, with both bound and unbound species of the probe being
25 contained within the sample. The irradiated sample emits emitted beam 18 which is detected at photomultiplier tube 20. Emitted beam 18 is amplitude modulated at the same frequency as the excitation but it is phase shifted and demodulated with respect to the excitation. It may be desirable to filter emitted beam 20 with
30 optical filter F in order to change the effective sensitivity range of the detector, as explained above. Typically, filter F is a 2-73 Corning filter, but any desired filter may be used, such as a Corning 3-67 filter.

Cross-correlation circuit 22 includes first frequency
35 synthesizer 24 which generates frequency f1, equal to one-half of a modulation frequency fM to drive acoustooptic modulator 14, and the PMT dynode chain. Cross-correlation circuit 22 also includes second frequency synthesizer 26 which generates a frequency f2

equal to the modulation frequency fM plus a cross-correlation frequency ▲f to drive photomultiplier tube 20. First frequency synthesizer 24 is coupled to frequency doubler 28, which directs a signal having a frequency equal to the modulation frequency fM to
5   mixer 30. Second, frequency synthesizer 26 also directs a signal having frequency f2 equal to the modulation frequency fM plus the cross-correlation frequency ▲f to mixer 30. Mixer 30 produces an output signal having a frequency equal to ▲f, the difference between fM and f2.
10   Mixer 30 and photomultiplier tube 20 are each connected to phase meter/digital voltmeter 32. Phase meter/digital voltmeter 32 compares the output signal having a frequency ▲f received from mixer 30 and the signal having a frequency ▲f(shifted) received from photomultiplier tube 20 to calculate the phase shift φ, and
15 the demodulation factor m which is stored in computer 34.

A.   Measuring pH and Carbon Dioxide Concentration

     As discussed above, a particular probe is selected to have intrinsic photoluminescent lifetime changes induced by the particular analyte to be measured. To measure pH, for example,
20 suitable probes include certain seminaphthorhodafluors, seminaphthofluoresceins (including BCECF acid) and resorufins. Specific example structures include:

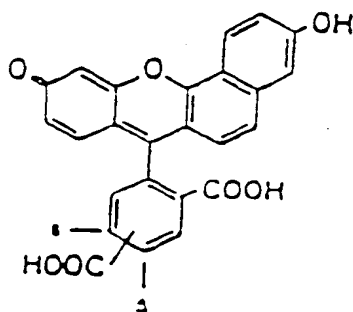SEMINAPHTHORHODAFLUORS
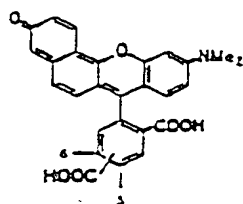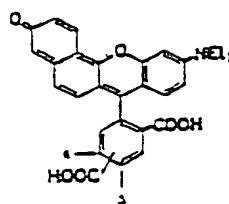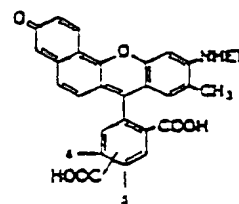
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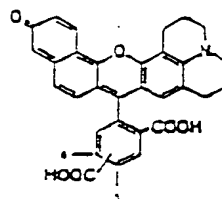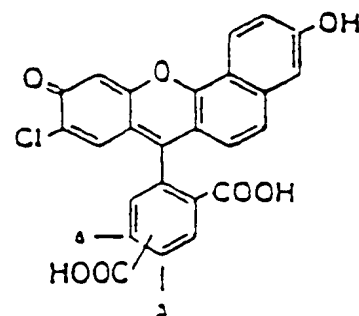

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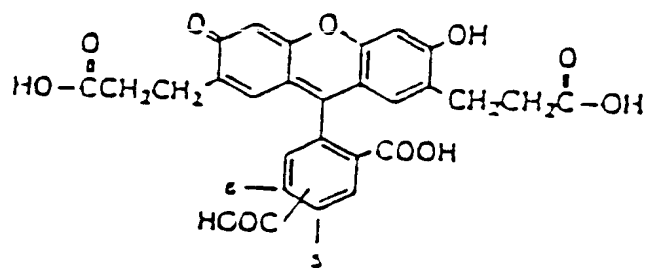
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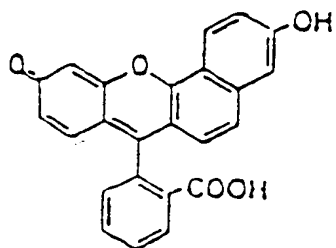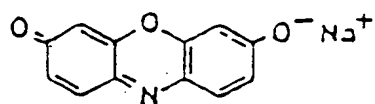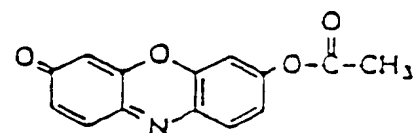

SNAFL-1

carboxy SNAFL-1

carboxy SNAFL-2

BCECF acid

RESORUFINS

Resorufin sodium salt

Resorufin acetate

These probes are available under the above-given tradenames
from Molecular Probes, Inc., Eugene, Oregon, and are commercially
sold as wavelength-shift pH indicators for intensity measurements.
The seminaphthorhodafluors and seminaphthofluoresceins are
5   disclosed in U. S. Patent No. 4,945,171, the contents of which is
incorporated herein by reference.

All of the following examples were taken in 80mM Tris at 25
degrees C.

Example 1

10   A sample containing the pH indicator Snafl-1 was irradiated
using a green helium-neon laser having an excitation wavelength of
543 nm modulated at a modulation frequency of 135 MHz. A Corning
2-73 filter was used to filter the emission beam. As shown in
Figure 1, at pH 7.24, the measured phase shift with respect to the
15   modulated excitation was 48.0 degrees, and the modulation of the
emission was 66.8% of the modulation of the excitation. In Figure
1, the phase is depicted as relative to its value at pH = 9.5 of
44.0 degrees with respect to the excitation. Similarly, the
modulation is depicted relative to its value at pH = 5.0 of 47.0%.

20   ## Example 2

A sample containing the pH indicator Carboxy Snafl-1 was
irradiated using a green helium-neon laser having an excitation
wavelength of 543 nm modulated at a modulation frequency of 135
MHz. A Corning 2-73 filter was used to filter the emission beam.
25   As shown in Figure 2, at pH 7.02, the measured phase shift with
respect to the modulated excitation was 46.1 degrees, and the
modulation of the emission was 61.0% of the modulation of the
excitation. In Figure 2, the phase is depicted as relative to its
value at pH = 9.5 of 43.0 degrees with respect to the excitation.
30   Similarly, the modulation is depicted relative to its value at pH
= 4.3 of 36.0%.

Example 3

A sample containing the pH indicator Carboxy Snafl-2 was
irradiated using a green helium-neon laser having an excitation
35   wavelength of 543 nm modulated at a modulation frequency of 135

MHz. Initially, a Corning 2-73 filter was used to filter the emission beam. As shown in Figure 3, at pH 7.10, the measured phase shift with respect to the modulated excitation was 43.6 degrees, and the modulation of the emission was 57.8% of the

5 modulation of the excitation.

As shown in Figure 17, further experimentation was conducted at various emission wavelengths. At pH = 7.10, the modulations with respect to the modulated excitations were 26.7% at 560 nm, 31.3% at 580 nm, 43.6% at 600 nm, 59.3% at 620 nm, 64.6% at 640 nm,

10 and 65.9% at 660 nm.

As shown in Figure 16, it is apparent that at pH = 7.0, for an excitation wavelength of 543 nm, the modulation is 43.6% with respect to the modulated excitation, whereas for an excitation wavelength of 560 nm, the modulation is 28.0%. Similarly, the

15 phase angle at an excitation wavelength of 543 nm, is 51.0 degrees and at 560 nm, is 82.0 degrees.

As is apparent from Figures 3, 16 and 17, the probe carboxy SNAFL-2 can be used in the method of the present invention to accurately measure pH in the range of 5 to 9, i.e., over a

20 concentration range of 4 decades.

Example 4

A sample containing the pH indicator BCECF acid was irradiated using a helium-cadmium laser having an excitation wavelength of 500 nm modulated at a modulation frequency of 65.0 MHz. As shown in

25 Figure 4, at pH 7.13, the measured phase shift with respect to the modulated excitation was 51.6 degrees, and the modulation of the emission was 62.7% of the modulation of the excitation. In Figure 4, the phase is depicted as relative to its value at pH = 4.2 of 49.0 degrees with respect to the excitation. Similarly, the

30 modulation is depicted relative to its value at pH = 9.5 of 52.0%.

At an excitation wavelength of 540 nm and a pH of 7.13, the measured phase shift was 58.7 degrees and the modulation was 51.4%. Again, the phase is depicted as relative to its value at pH = 4.2 of 50.0 degrees with respect to the excitation, and the modulation

35 is depicted relative to its value at pH = 9.5 of 47.0%.

Example 5

A sample containing the pH indicator Carboxy Snarf-1 was
irradiated using a green helium-neon laser having an excitation
wavelength of 543 nm modulated at a modulation frequency of 135
5   MHz.   A Corning 2-73 filter was used to filter the emission beam.
As shown in Figure 5, at pH 6.91, the measured phase shift with
respect to the modulated excitation was 36.5 degrees, and the
modulation of the emission was 70.0% of the modulation of the
excitation.   The phase is depicted as relative to low pH and the
10   modulation as relative to high pH.

As can be seen from Figure 12, the difference in phase angles
observed for this probe in the bound and unbound forms is maximal
in the range of about 135 MHz; thus a modulation frequency in this
range is preferred because it provides optimum precision and
15   dynamic range.

Example 6

A sample containing the pH indicator Carboxy Snarf-2 was
irradiated using a green helium-neon laser having an excitation
wavelength of 543 nm modulated at a modulation frequency of 135
20   MHz.   Initially, a Corning 2-73 filter was used to filter the
emission beam.   As shown in Figure 6, at pH 7.0, the measured phase
shift with respect to the modulated excitation was 27.0 degrees,
and the modulation of the emission was 65.0% of the modulation of
the excitation.   In Figure 6, the phase is depicted as relative to
25   its value at pH = 4.9 of 10.0 degrees with respect to the
excitation.   Similarly, the modulation is depicted relative to its
value at pH = 9.2 of 58.0%.

Example 7

A sample containing the pH indicator Carboxy Snarf-6 was
30   irradiated using a green helium-neon laser having an excitation
wavelength of 543 nm modulated at a modulation frequency of 135
MHz.   Initially, a Corning 2-73 filter was used to filter the
emission beam.   As shown in Figure 7, at pH 6.92, the measured
phase shift with respect to the modulated excitation was 59.8
35   degrees, and the modulation of the emission was 34.6% of the
modulation of the excitation.   The phase is depicted relative to

its high pH value of 41.7 degrees, and the modulation is depicte relative to its low pH value of 25.3%.

Example 8

A sample containing the pH indicator Carboxy Snarf-X wa
5 irradiated using a green helium-neon laser having an excitatio
wavelength of 543 nm modulated at a modulation frequency of 13
MHz. A Corning 2-73 filter was used to filter the emission beam.
As shown in Figure 8, at pH 6.88, the measured phase shift with
respect to the modulated excitation was 54.0 degrees, and the
10 modulation of the emission was 53.6% of the modulation of the
excitation. In Figure 8, the phase is depicted as relative to its
value at pH = 4.8 of 51.0 degrees with respect to the excitation.
Similarly, the modulation is depicted relative to its value at pH
= 9.5 of 42.0%.

15
Example 9

A sample containing the pH indicator Resorufin Sodium Salt
was irradiated using a helium-cadmium laser having an excitation
wavelength of 442 nm modulated at a modulation frequency of 135
MHz. Initially, a Corning 3-67 filter was used to filter the
20 emission beam. As shown in Figures 9A and 9B, at pH = 6.1, the
measured phase shift with respect to the modulated excitation was
58.1 degrees, and the modulation of the emission was 41.1% of the
modulation of the excitation. At an emission wavelength of 560
nm, the measured phase shift with respect to the modulated
25 excitation was 41.1 degrees, and the modulation of the emission
was 45.3% of the modulation of the excitation. At an excitation
wavelength of 543 nm and a pH of 3.5, the measured phase shift with
respect to the modulated excitation was 29.8 degrees, and the
modulation of the emission was 62.6% of the modulation of the
30 excitation. At an excitation wavelength of 566 nm and a pH of
2.67, the measured phase shift with respect to the modulated
excitation was 39.5 degrees, and the modulation of the emission
was 55.0% of the modulation of the excitation.

Example 10

35   A sample containing the pH indicator Resorufin Acetate was

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irradiated using a helium-cadmium laser having an excitation
wavelength of 442 nm modulated at a modulation frequency of 135
MHz. Initially, a Corning 3-67 filter was used to filter the
emission beam. As shown in Figure 10, at pH 5.52, the measured
5 phase shift with respect to the modulated excitation was 41.0
degrees, and the modulation of the emission was 50.4% of the
modulation of the excitation. In Figure 10, the phase is depicted
as relative to its value at pH = 3.1 of 16.4 degrees with respect
to the excitation. Similarly, the modulation is depicted relative
10 to its value at pH = 7.5 of 38.1%.

                            Example 11
        A sample containing the indicator carboxy SNARF-6 in hydrogel
was irradiated using a green helium-neon laser having an excitation
wavelength of 543 nm modulated at a modulation frequency of 135
15 MHz. A Corning 2-73 filter was used to filter the emission. As
shown in Figures 13A and 13B, at 4% carbon dioxide (expressed as a
percentage of total gas molecules overlaying the sample, with the
balance being nitrogen), the measured phase shift with respect to
the modulated excitation was 71.1 degrees, and the modulation of
20 the emission was 27.5% of the modulation of the excitation.

                    *   *   *   *   *

        All of the probes in Examples 1-11 are excitable with a 543
nm green helium-neon laser, except for BCECF acid. The use of a
green helium-neon laser is particularly advantageous in the
25 clinical setting because the laser is inexpensive and reliable.
        These probes also exhibit acceptable phase and modulation
changes. The response for BCECF acid is the smallest, and the
response for resorufin sodium salt is the largest. As discussed
above, phase shifts on the order of 30-60 degrees are preferred.
30 Accordingly, of the above pH probes, carboxy snafl-2 and carboxy
snarf-6 exhibit the particularly useful phase and modulation
changes.
        The results are dependent on the excitation and emission
wavelengths, since these variables cause either the bound or

unbound form of the probe to be emphasized, as explained above.
For pH measurements, this allows the range of pH sensitivity to be
selected depending on the choice of excitation wavelength or
emission wavelength.

5   Some of the above probes are more dependent on these variables
than others. For example, of the seminaphthofluoresceins, carboxy-
snafl-2 appears to be the best at allowing measurements of various
pH ranges by changing the excitation and emission wavelengths. Of
the seminaphthorhodafluors, carboxy snarf-6 appears to be the best
10 in this respect.   Although both of the resorufins show large
changes in phase and modulation, resorufin sodium salt appears to
allow greater range of pH sensitivity than resorufin acetate.

        The method of the invention is particularly useful in a
clinical setting for measuring blood gases quickly and effectively.
15 For example, the pH of a blood sample can be measured and certain
blood   gases,   such   as   carbon   dioxide   concentration,   can   be
determined therefrom. This method of determining the pH and carbon
dioxide concentration of a blood sample can be used in vitro or in
vivo including, for example, blood gas catheters and other bedside
20 patient monitors, and non-invasive blood gas measurements.

B.   Measuring Calcium Ion Concentration

        The method of the invention may also be used to measure the
calcium ion concentration of a sample.   To measure calcium ion
concentration, certain tetraacetic acids, particularly Quin-2 (2-
25 [(2-amino-5-methylphenoxy)   methyl]-6-methoxy-8-amino-quinoline-
N,N,N',N'-tetraacetic acid, tetrapotassium salt) having the
following structure has proven particularly useful:
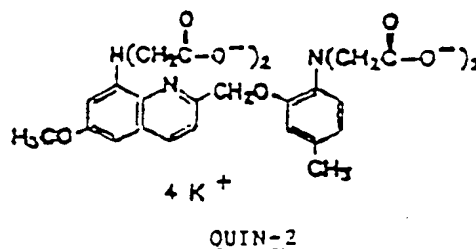
QUIN-2

The short lifetime component shown in Figure 11 corresponds to the
probe bound to the calcium ions, and the long lifetime component
30 corresponds to the free probe.

Example 12

A sample containing the calcium ion indicator Quin-2 in a buffered solution of 130 mM KCl desolved in 10 mM MOPS and 1 mM EDTA at a pH of 7.06 was irradiated using a laser having an 5 excitation wavelength of 293 nm modulated at a frequency of 49.53 MHz. The emission wavelength was 500 nm. As shown in Figure 11, at a calcium ion concentration of 26 nM, the measured phase shift with respect to the modulated excitation was 45.1 degrees, and the modulation of the emission was 40.0% of the modulation of 10 the excitation. At a calcium ion concentration of 26 nM and an excitation wavelength of 346 nm, the measured phase shift with respect to the modulated excitation was 32.0 degrees, and the modulation of the emission was 54.2% of the modulation of the excitation.

15   *   *   *   *   *

As can be seen from the above, as was found in Examples 1-11, the results in Example 12 are dependent on the excitation and emission wavelengths, since these variables cause either the bound or unbound form of the probe to be emphasized, as explained above. 20 For calcium ion concentration measurements, this allows the range of calcium ion concentration sensitivity to be selected depending on the choice of excitation wavelength and/or emission wavelength.

It is also contemplated that the probes known under the tradenames Fura-2, Indo-1, Rhod-2, Fluo-3, Calcium Green, Calcium 25 Orange and Calcium Crimson may be suitable for making calcium ion concentration measurements using the method of the present invention.

Sensing the calcium ion concentration of a sample is particularly useful for flow cytometry and fluorescent lifetime 30 imaging applications.

C.   Measuring Potassium Ion Concentration

The method of the invention may also be used to measure the potassium ion concentration of a sample. To measure potassium ion

concentration, the probe known as PBFI, having the following structure, has been found to be particularly useful:
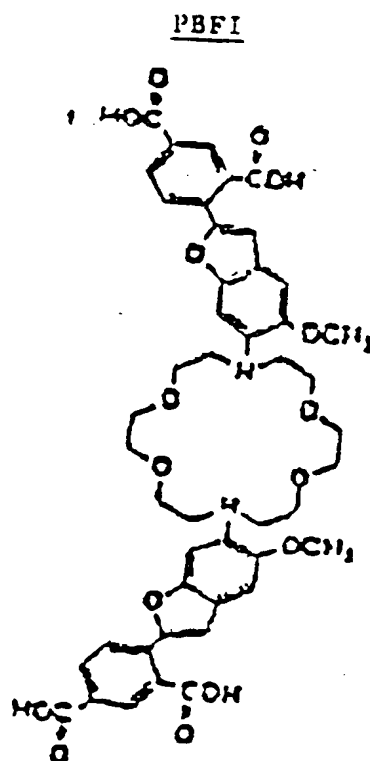
PBFI

Example 13

A sample containing the potassium ion indicator PBFI was
5 irradiated using a laser having an excitation wavelength of 355 nm

modulated at a frequency of 975.3 MHz.   As shown in Figure 14, at
a potassium ion concentration of 200 mM, the measured phase shift
with respect to the modulated excitation was 57.5 degrees.   In
Figure 14, the phase is depicted as relative to its value at a
5 potassium ion concentration of 0 mM of 48.1 degrees with respect to
the excitation.   At a modulation frequency of 193.6 MHz and a
calcium ion concentration of 200 mM, the measured phase shift with
respect to the modulated excitation was 31.2 degrees.   The phase is
depicted as relative to its value at a potassium ion concentration
10 of 0 mM of 26.3 degrees with respect to the excitation.

                    *   *   *   *   *

        The above is for illustrative purposes only. Modifications
can be made within the scope of the invention as defined by the
appended claims.

We claim:

1. A method of optically measuring analytes, said method comprising the steps of:

adding a photoluminescent ligand probe to a sample to be analyzed, said probe having intrinsic analyte-induced lifetime changes;

exciting the sample with radiation;

detecting the resulting emission beam; and

calculating the apparent luminescence lifetime to determine the analyte concentration of the sample.

2. A method as in claim 1, wherein the analyte is hydronium ions.

3. A method as in claim 2, further comprising the step of calculating carbon dioxide concentration from the hydronium ion concentration.

4. A method as in claim 3, wherein the carbon dioxide concentration is calculated through the bicarbonate couple to pH.

5. A method as in claim 1, wherein said probe is a phosphorescent probe.

6. A method as in claim 1, wherein said probe is a fluorescent probe.

7. A method as in claim 1, wherein the sample is human blood or a fraction thereof.

8. A method as in claim 2, wherein said probe is selected from the group consisting of seminaphthorhodafluors, seminaphthofluoresceins and resorufins.

9. A method as in claim 7, wherein said probe is selected from the group consisting of seminaphthorhodafluors, seminaphthofluoresceins and resorufins.

10. A method as in claim 2, wherein said probe is selected from the group consisting of the following formulae:

SEMINAPHTHOFLUORESCEINS
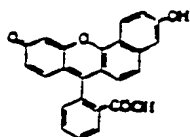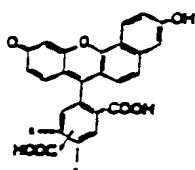
SNAFL-1

carboxy SNAFL-1

carboxy SNAFL-2

BCECF acid
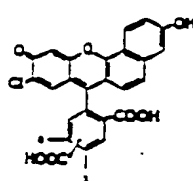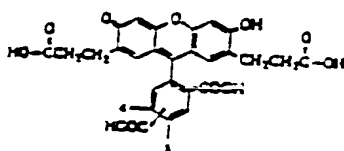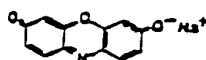
RESORUFINS

Resorufin sodium salt
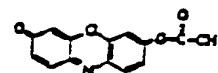
Resorufin acetate

SEMINAPHTHORHODAFLUORS
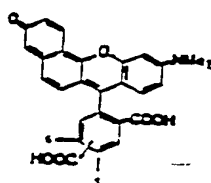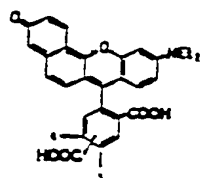
carboxy SNARF-1
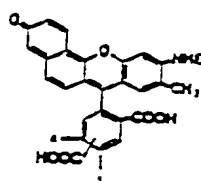
carboxy SNARF-2
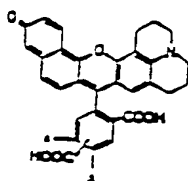
carboxy SNARF-6

carboxy SNARF-X

11. A method as in claim 1, wherein said method is used fo the continuous in-vivo monitoring of blood gases.

12. A method as in claim 1, wherein the lifetime i calculated using phase-modulation fluorometry.

13. A method as in claim 1, further comprising the step o changing the apparent concentration sensitivity range of the prob by changing the wavelength of the modulated excitation and/or th wavelength of the emission.

14. A method as in claim 1, wherein the analyte is ionic calcium.

15. A method as in claim 14, wherein said probe has the following formulae:
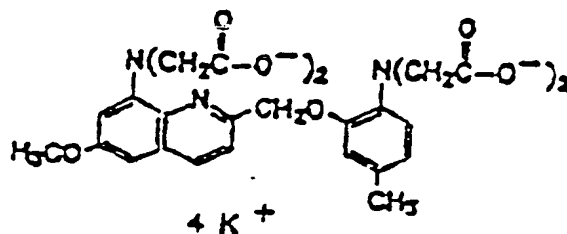
QUIN-2

16. A method as in claim 1, wherein the method is used for flow cytometry.

17. A method as in claim 1, wherein the method is used for fluorescent lifetime imaging.

18. A method as in claim 14, wherein the excitation wavelength is 293 nm.

19. A method as in claim 14, wherein the excitation wavelength is 346 nm.

SUBSTITUTE

20. A method as in claim 14, wherein the excitation
wavelength is 357 nm.

21. A method as in claim 14, wherein a Corning 3-72 filter
is used to filter the emission beam.

22. A method as in claim 1, wherein the analyte is ionic
potassium.

23. A method as in claim 22, wherein the probe has the
following formula:

PBFI
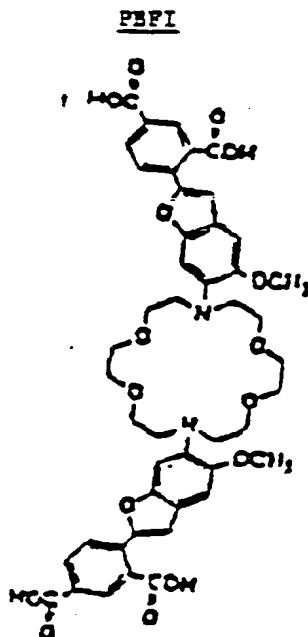
24. A method as in claim 22, wherein the excitation
wavelength is 355 nm.

25.   A method as in claim 1, wherein the lifetime is
calculated using time-resolved fluorometry.

26. A method as in claim 1, wherein the sample is excited
using a green helium-neon laser.

27.   A method as in claim 1, wherein the sample is excited using a laser diode.

28.   A method as in claim 27, wherein the laser diode is intrinsicly modulated.
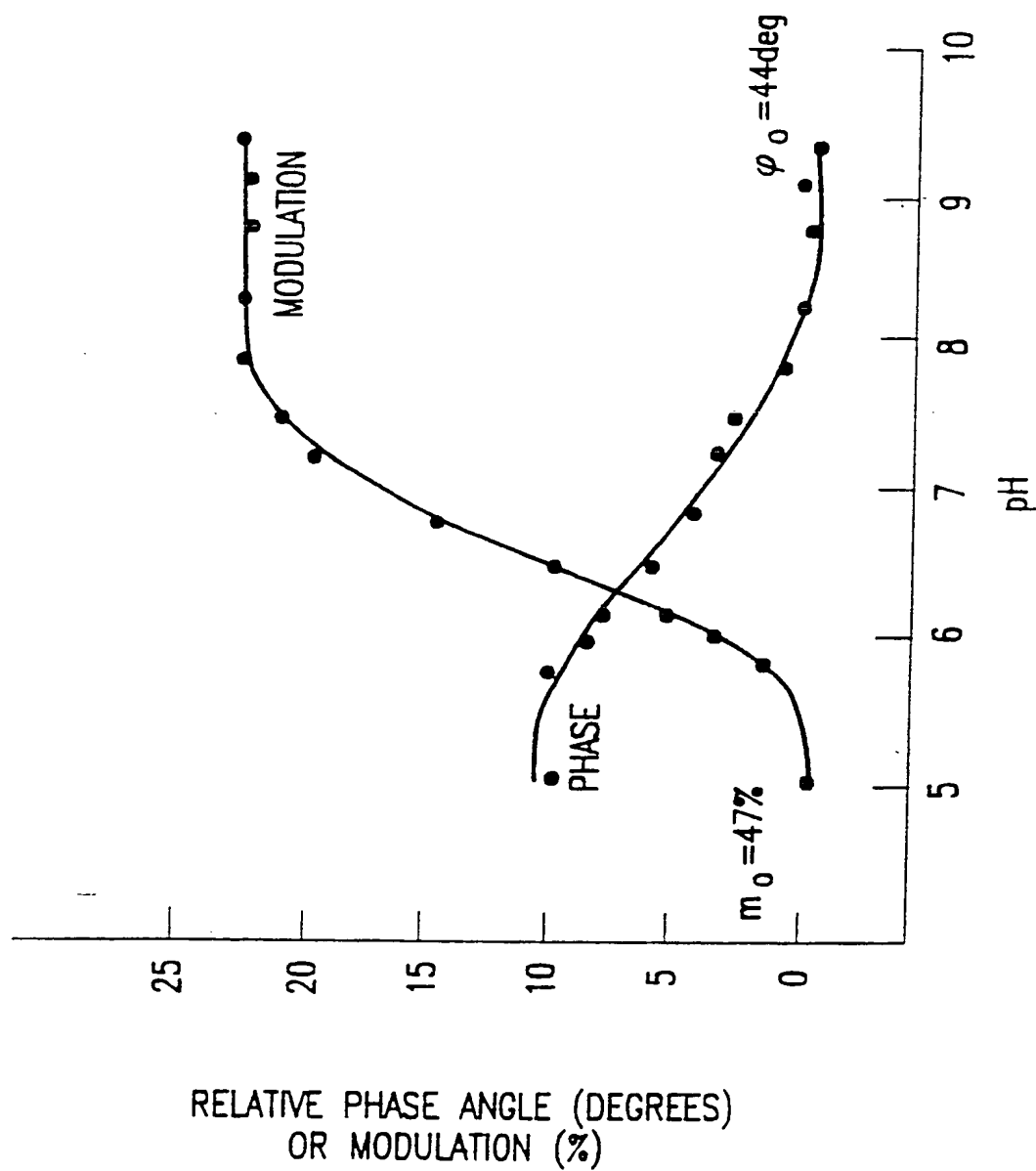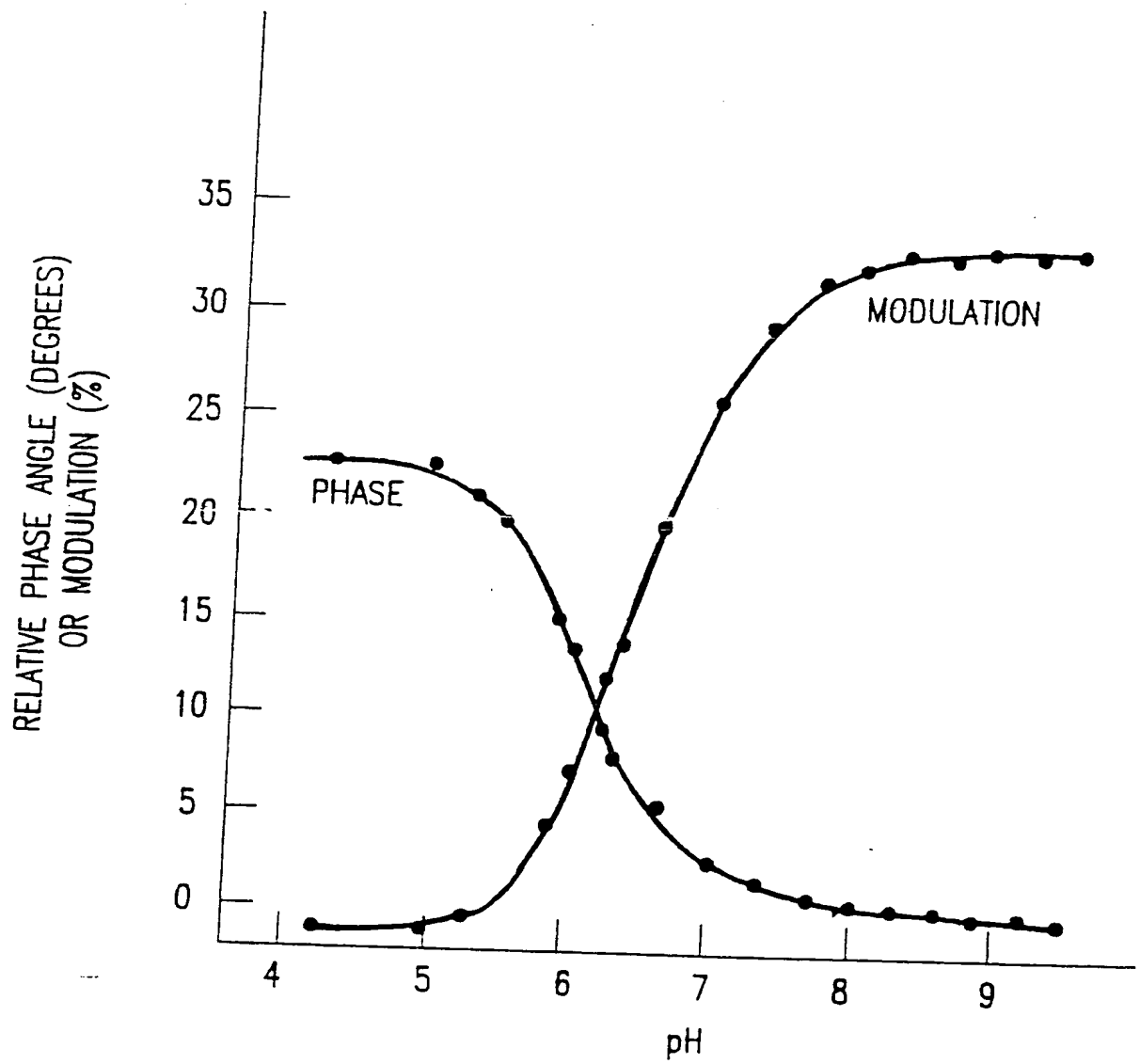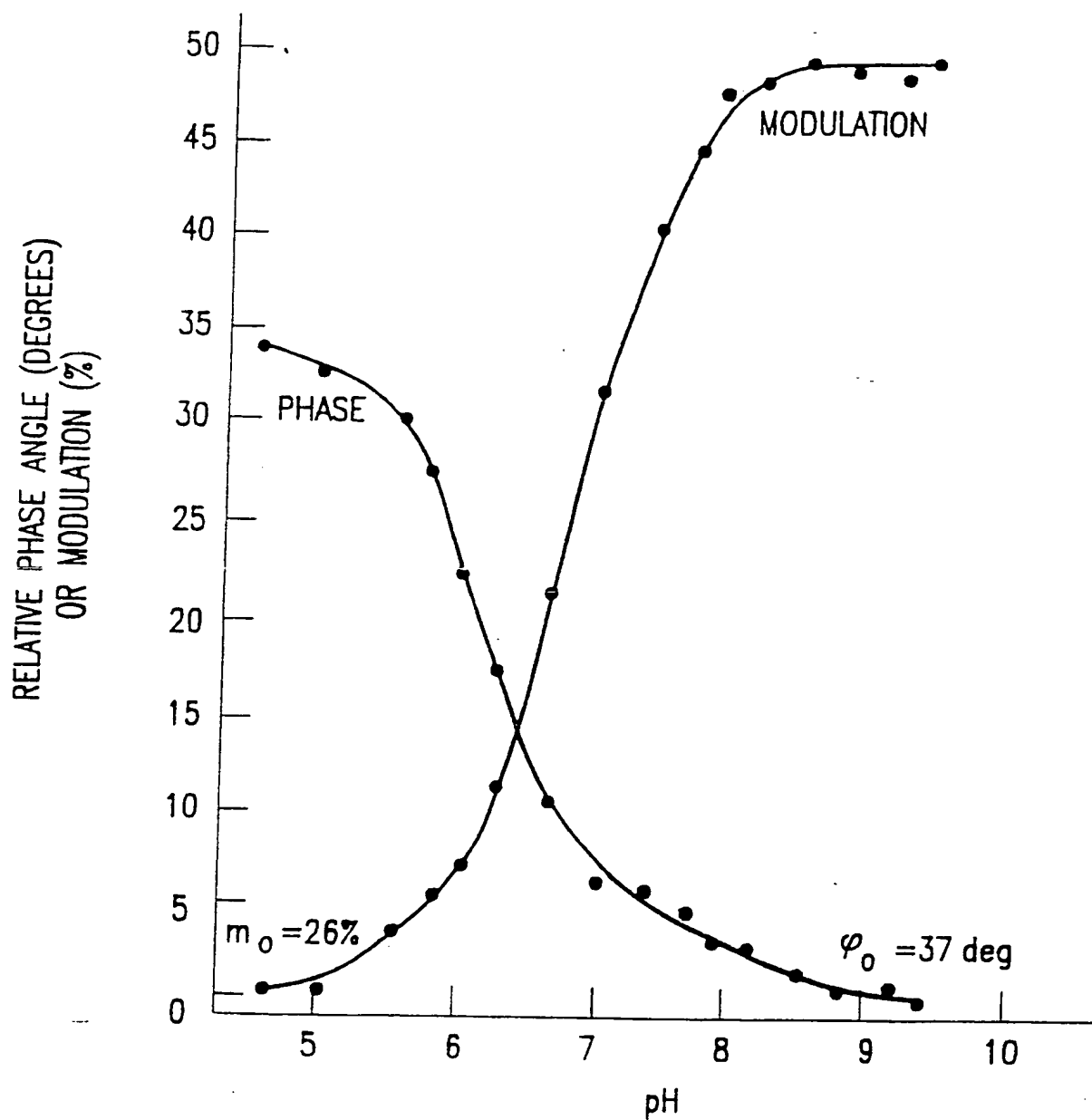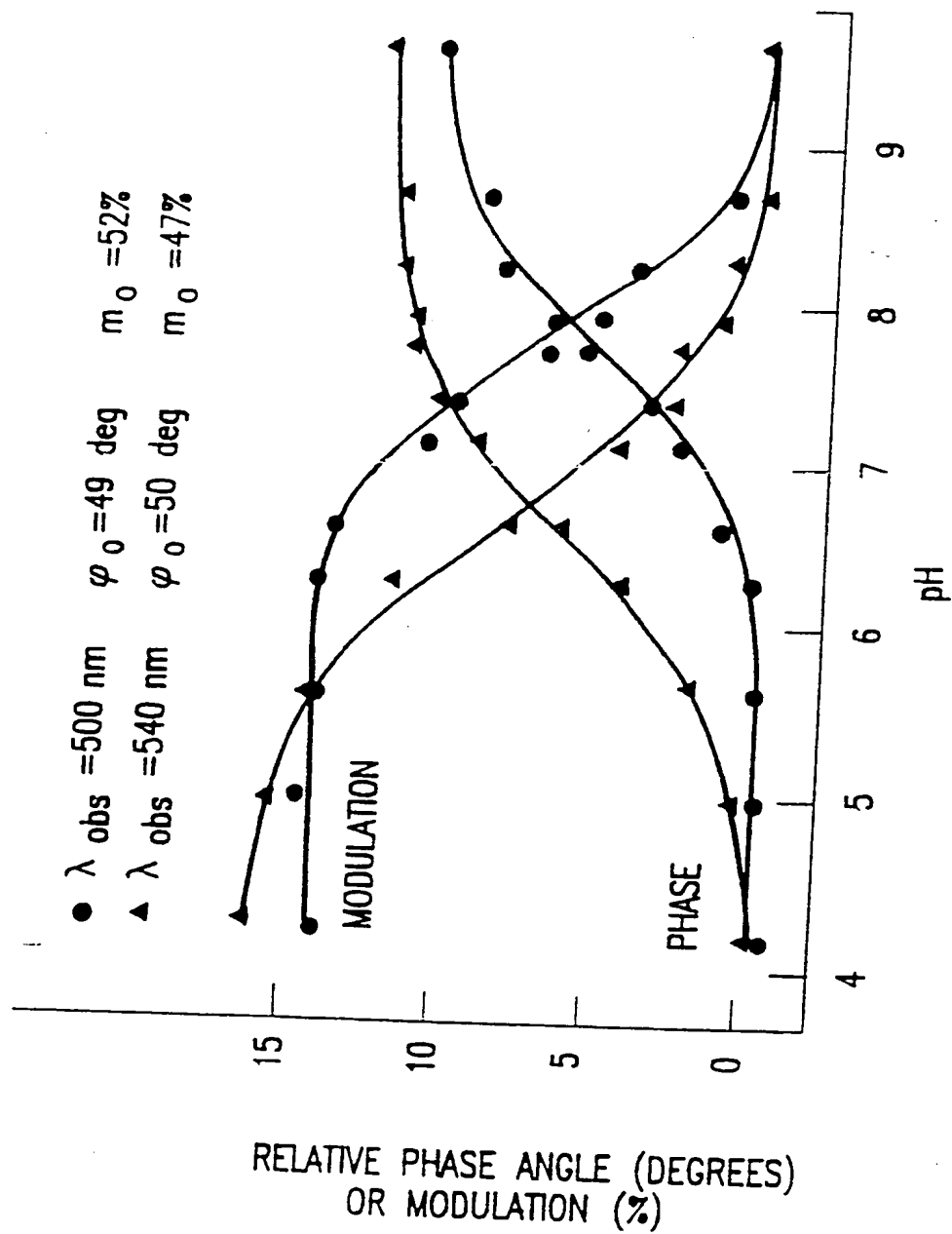
FIG.1

FIG.2
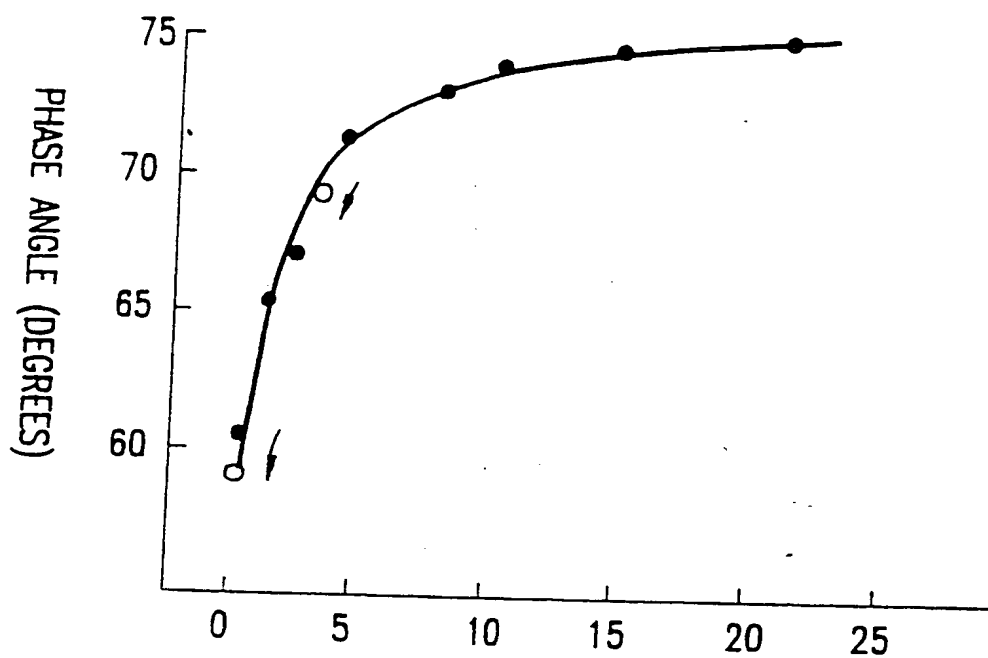
FIG.3

FIG.4
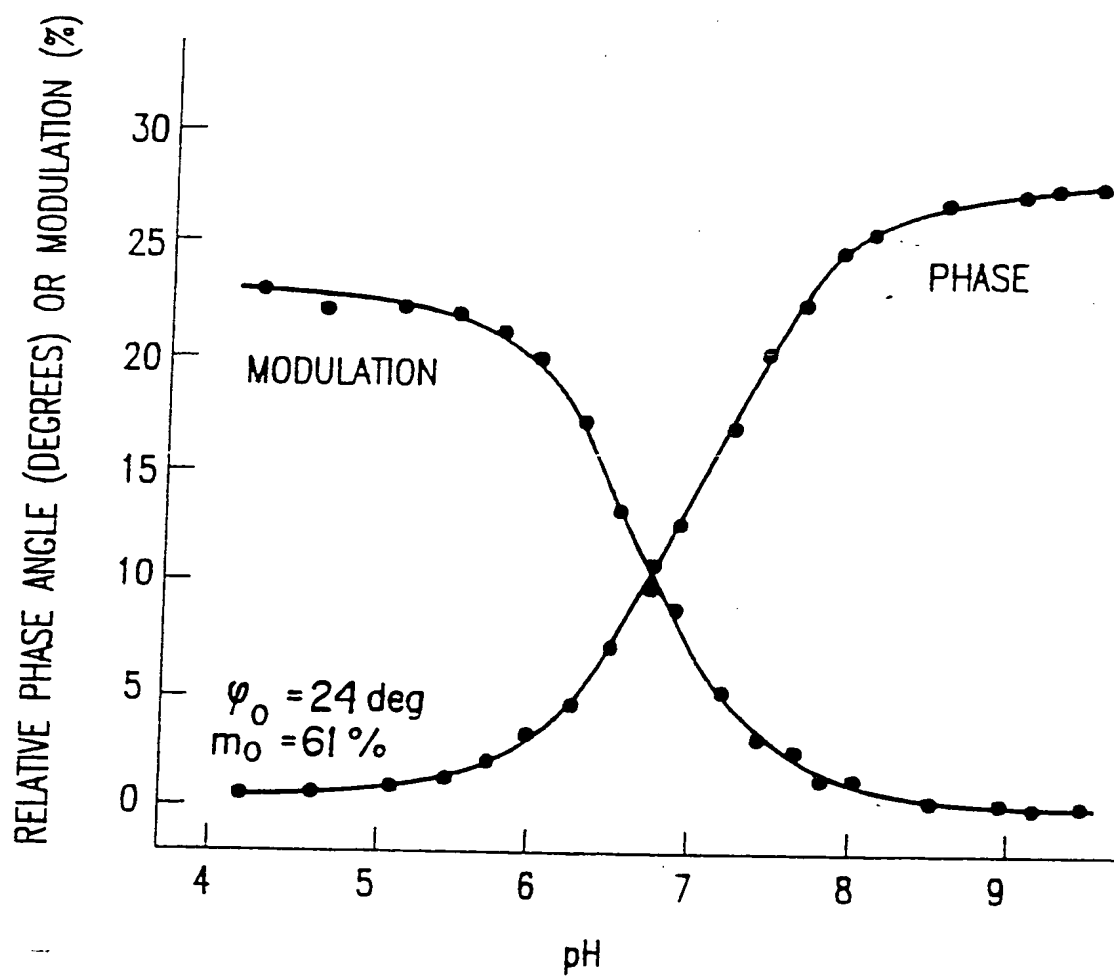
FIG.5
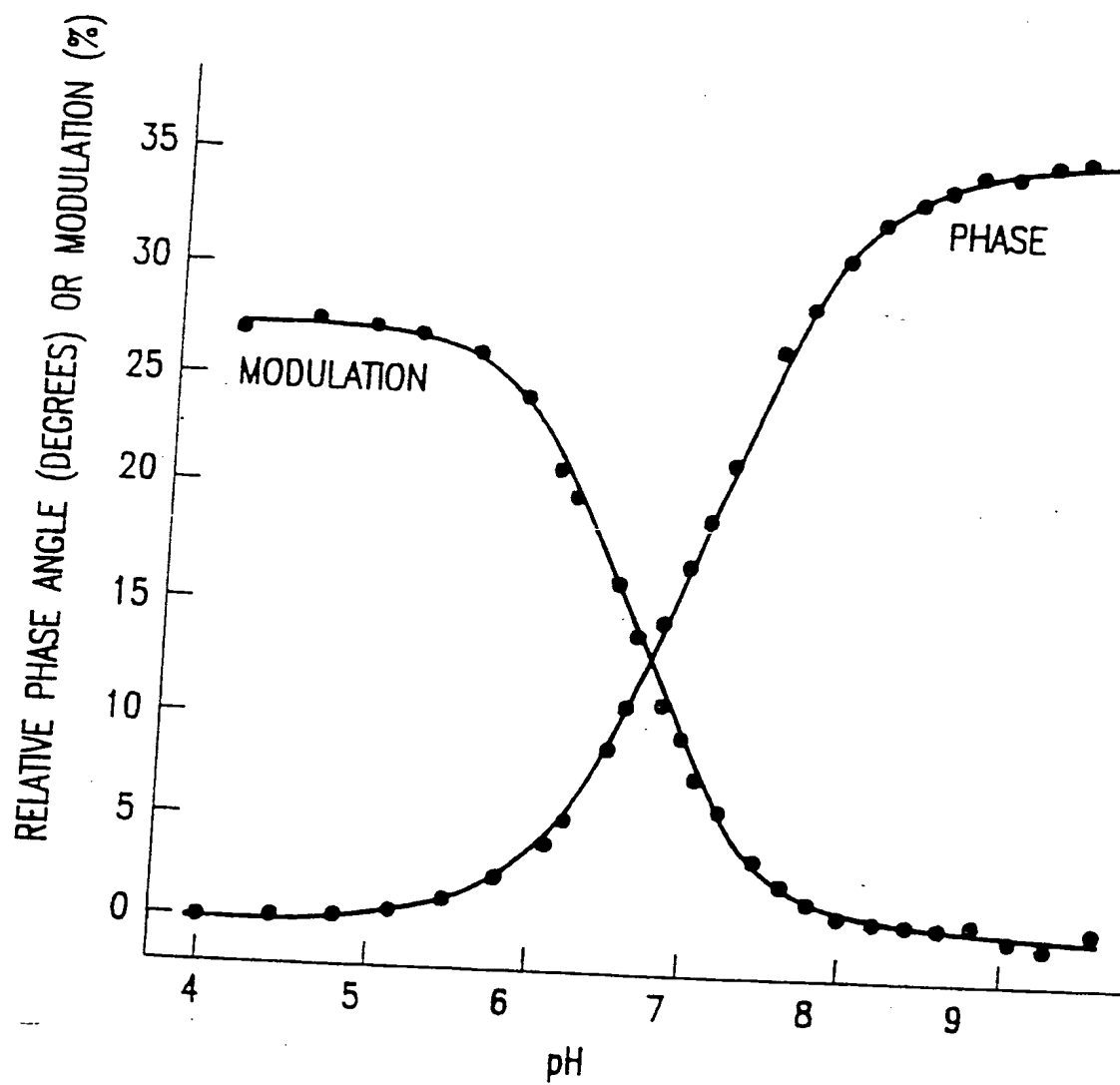
FIG.6
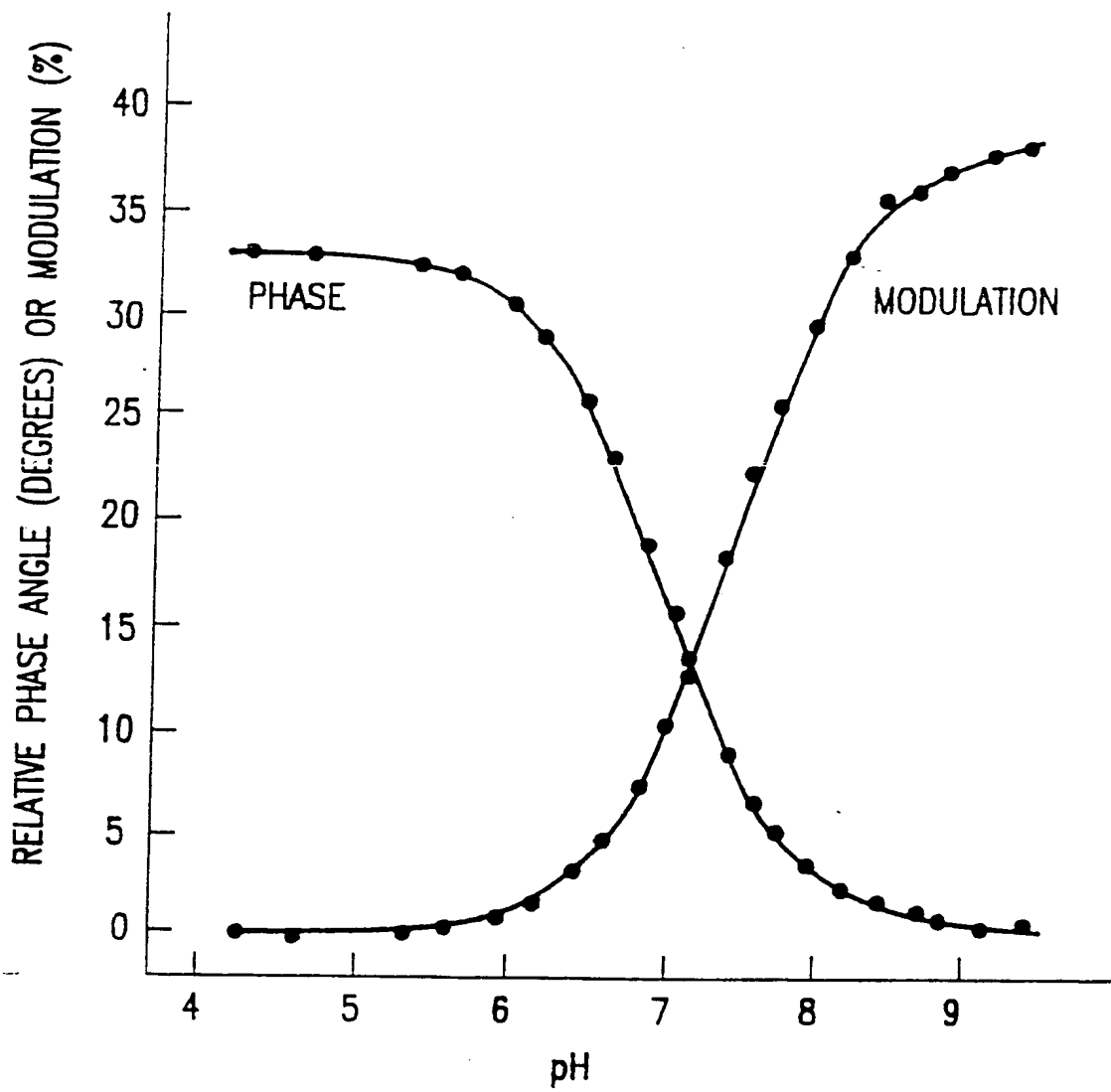
FIG.7
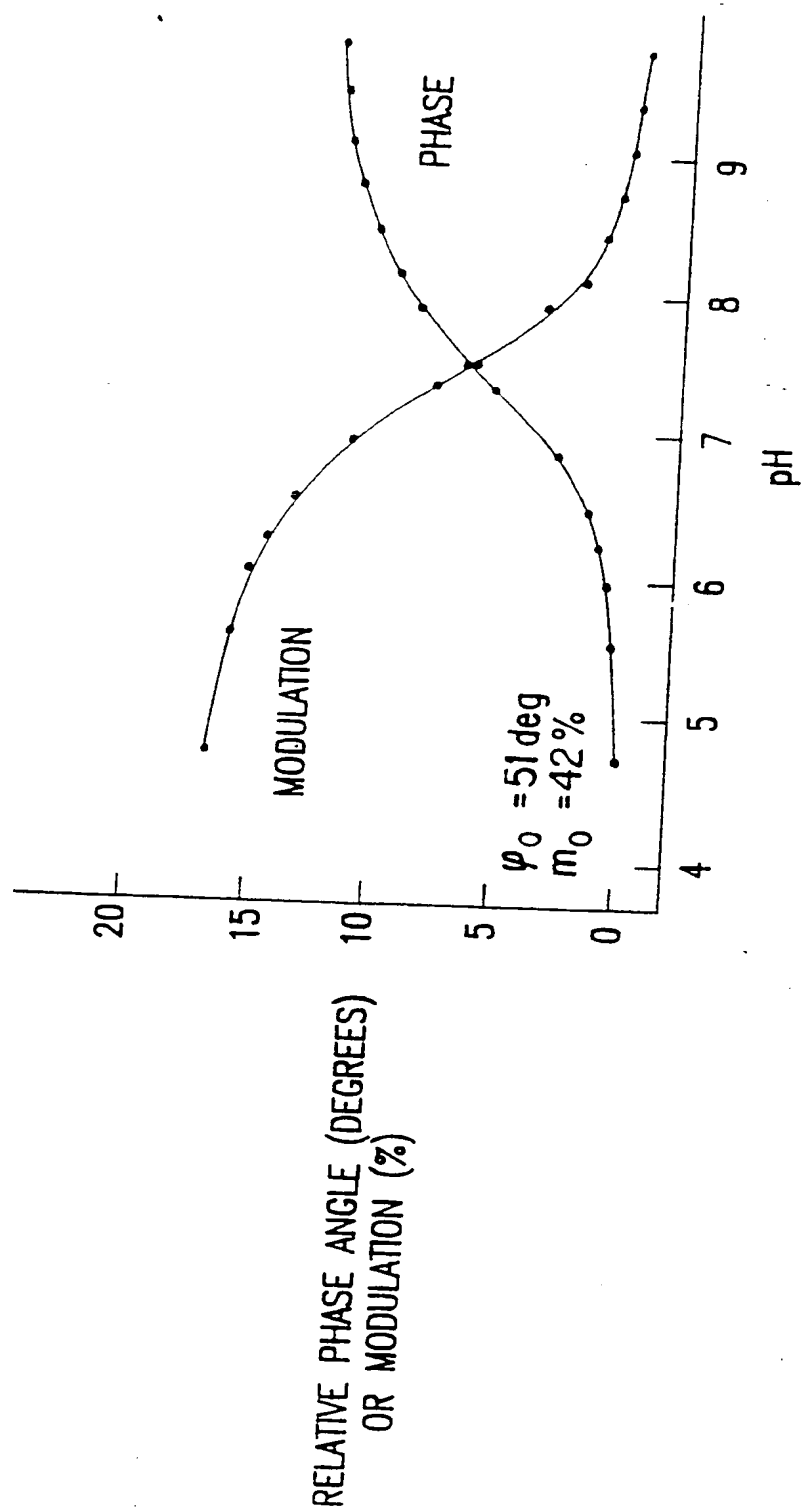
FIG.8
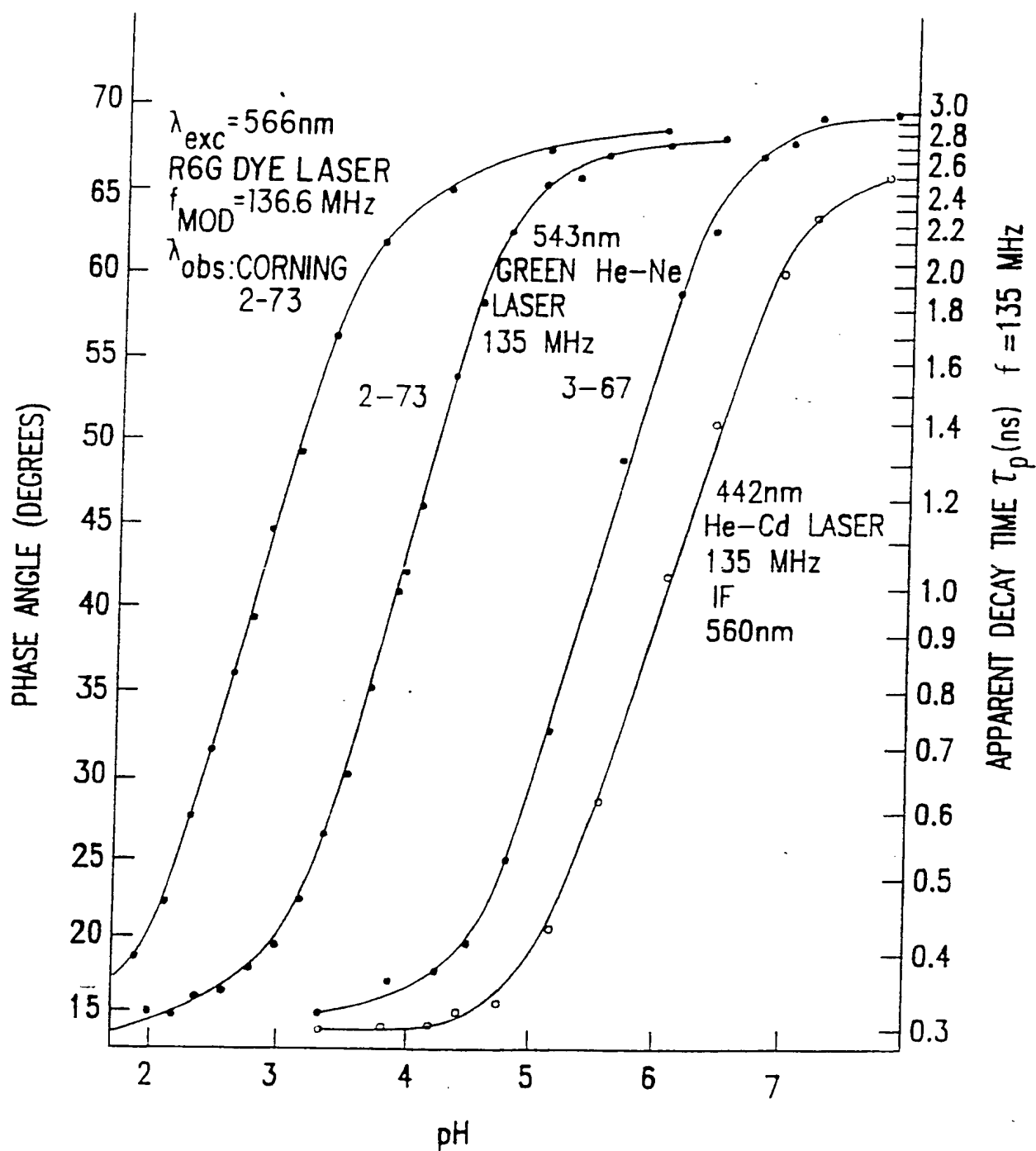
FIG.9A
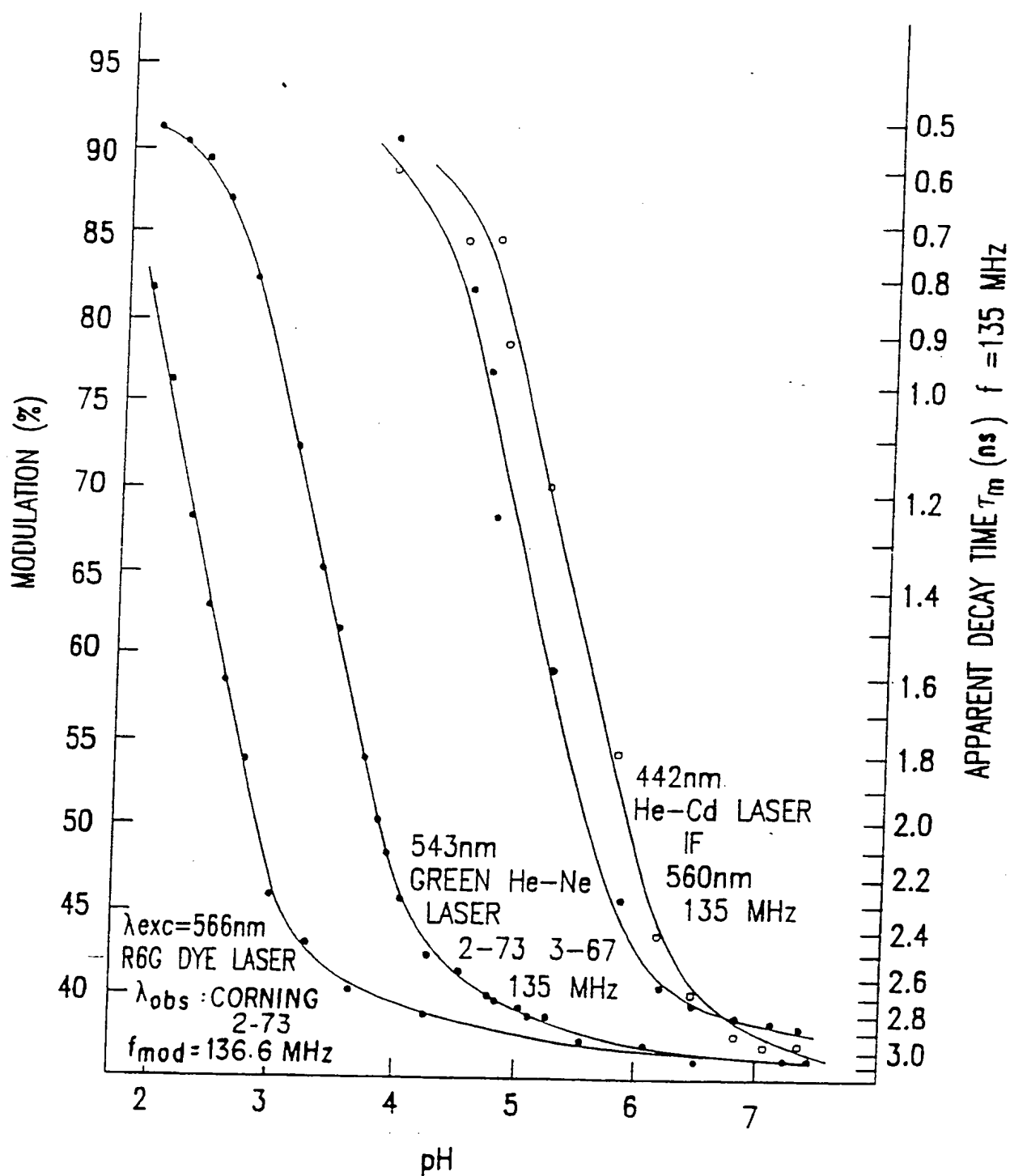
FIG.9B
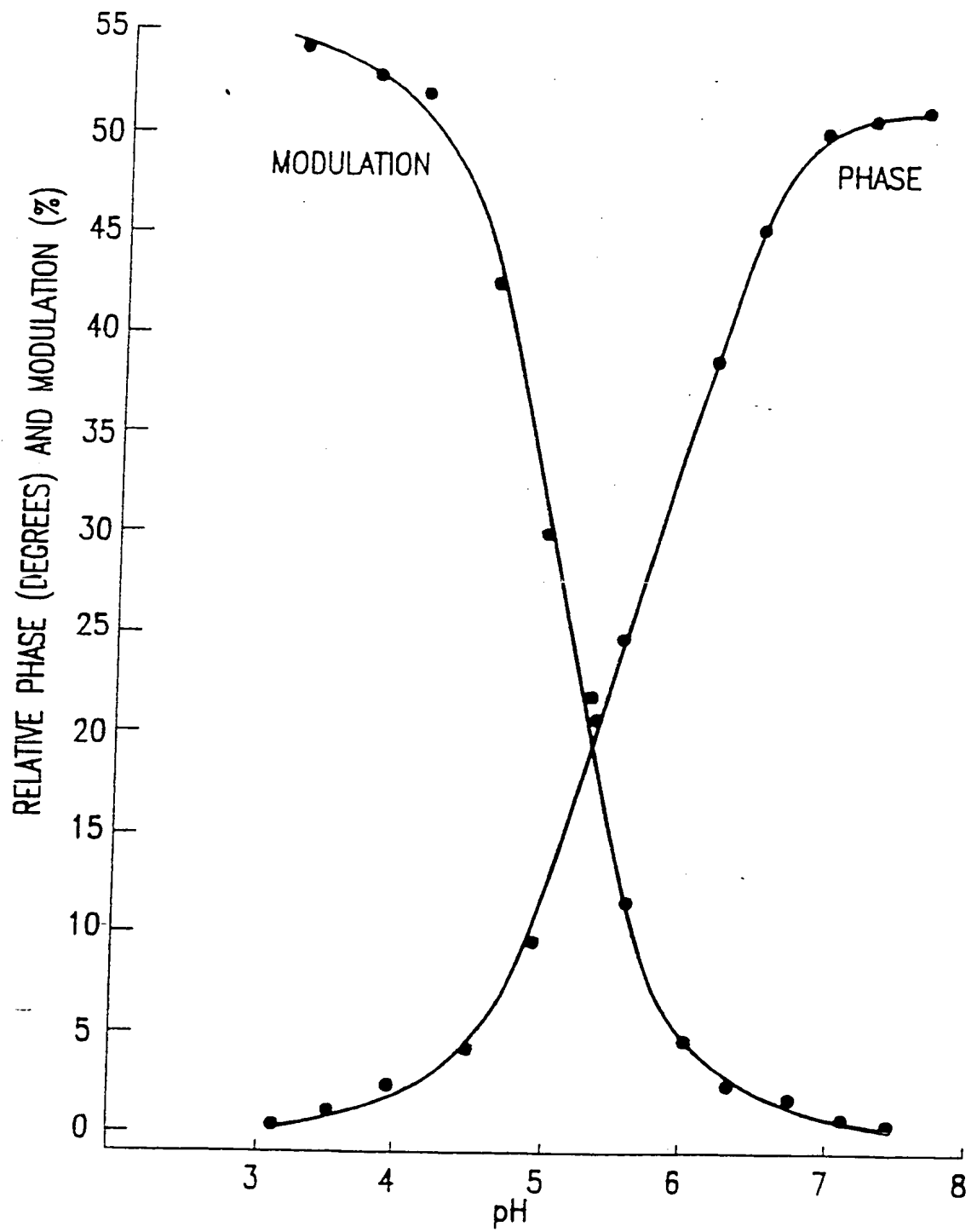
FIG.10
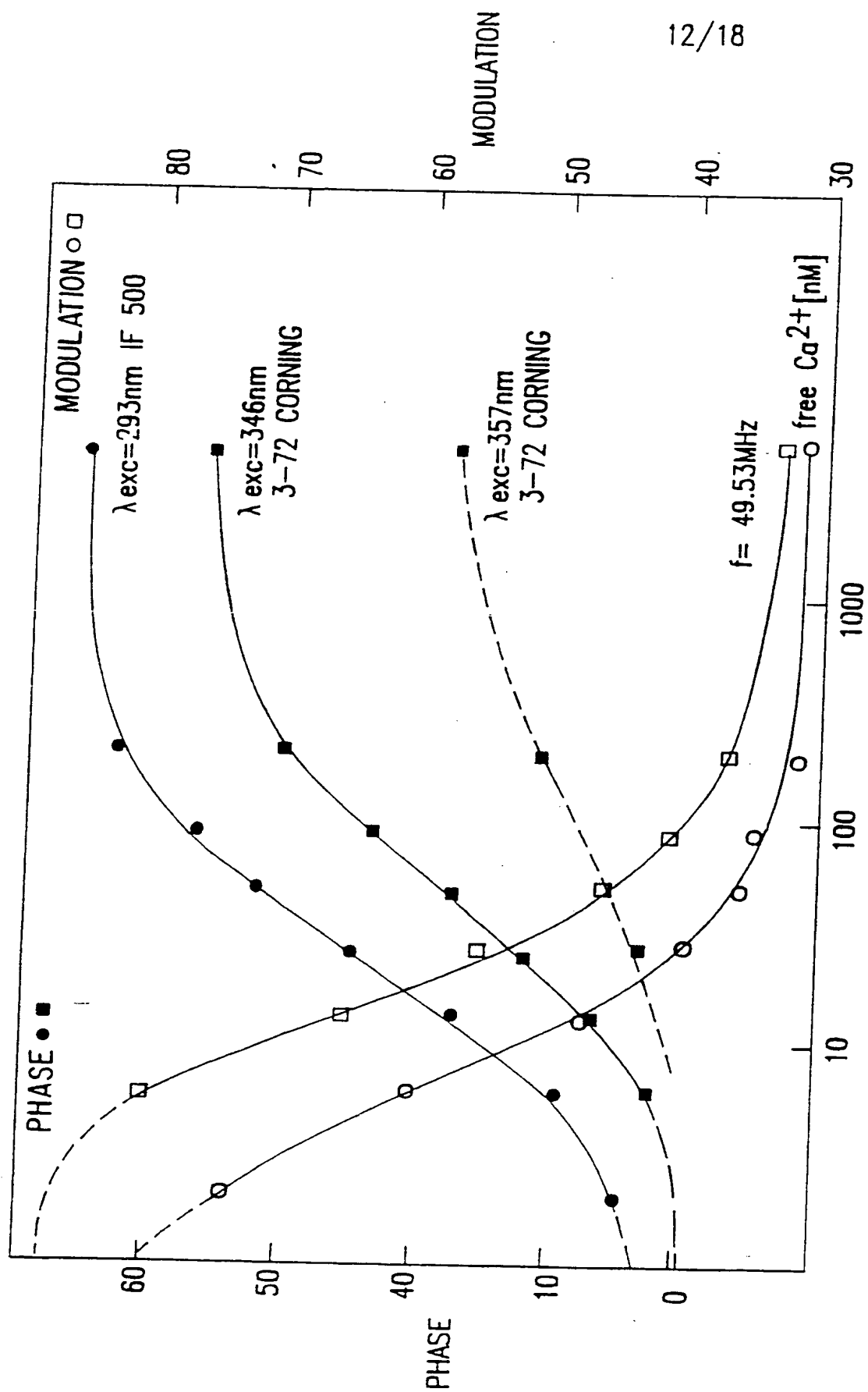
FIG.11
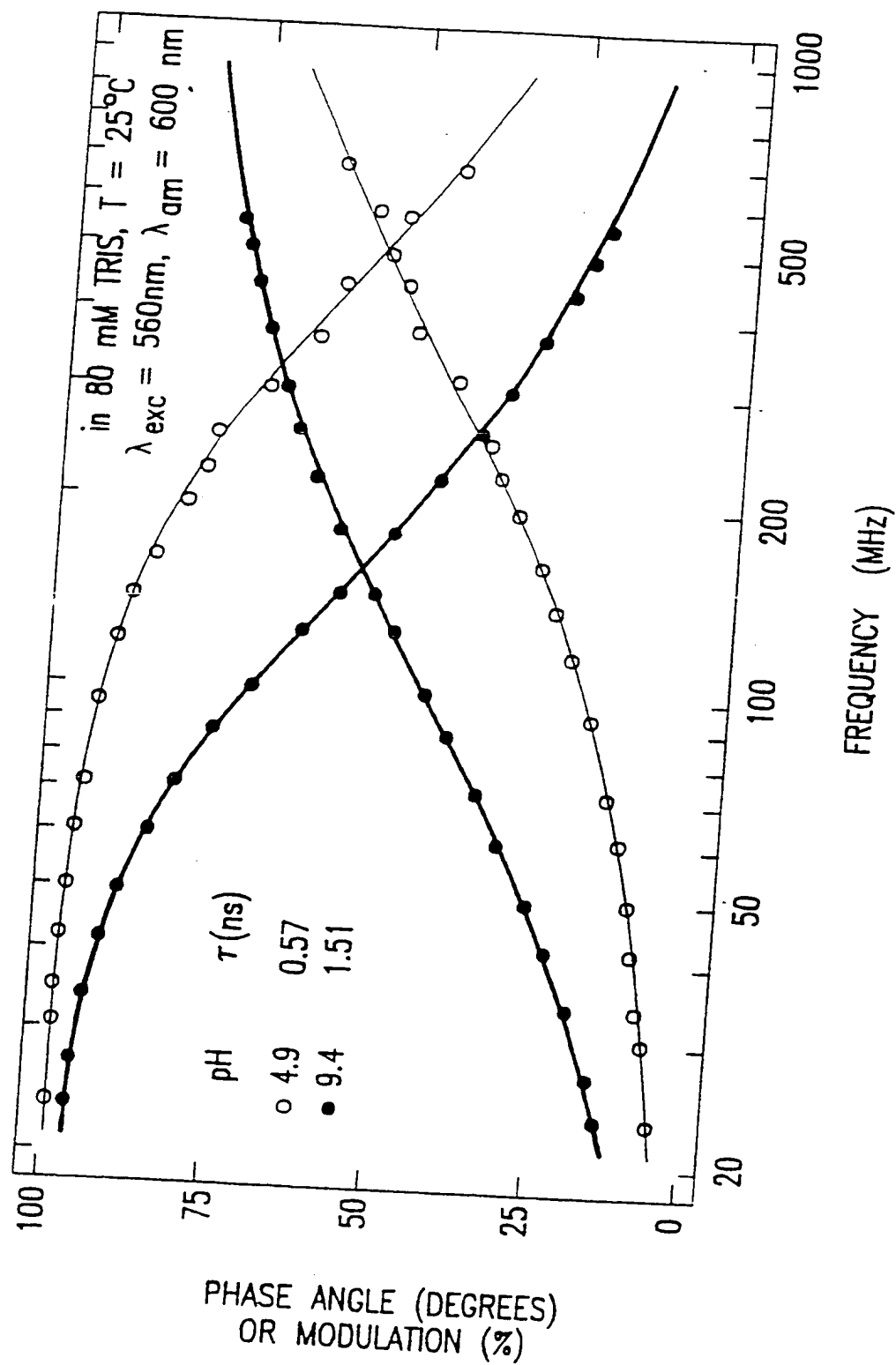
FIG.12

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FIG.13A

FIG.13B

FIG.14

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FIG.15

FIG.16

FIG.17